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Immunofluorescence and Immunoelectron Microscopic Studies in Cicatricial Pemphigoid

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We have studied various tissues from 10 patients with cicatricial pemphigoid using direct and indirect immunofluorescence, mechanical suction blister induction, and immunoelectron microscopy. In 8 of the 10 patients, direct immunofluorescence of buccal mucosa showed a linear deposition of immunoreactants, IgG and C3 being those most commonly detected. Direct immunofluorescence of skin was positive in only 4 patients. Only 1 patient had a detectable circulating anti-basement membrane zone antibody. Substitution of normal human oral mucosa for adult skin as the tissue substrate for indirect immunofluorescence did not prove useful in the detection of circulating autoantibodies. Immunoelectron microscopy was performed in the skin or mucosa (buccal or ocular) of 6 patients, revealing lamina lucida localization of in vivo-bound immunoreactants. Indirect immunofluorescence studies on mechanically induced suction blisters in skin of 2 patients with in vivo-bound IgG suggest that the lamina lucida antigen involved in cicatricial pemphigoid may be distinct from the bullous pemphigoid antigen.

Cicatricial pemphigoid is a chronic scarring bullous disease primarily involving mucous membranes and less frequently skin. Most patients with this disease have in vivo-bound immunoglobulins and/or complement along the basement membrane zone of affected tissues. Less frequently, sera from these patients may contain circulating anti-basement membrane zone autoantibodies.

Prior to the present study, only 2 patients have been reported in whom ultrastructural localization of in vivo-bound immunoreactants was performed [1,2]. In both of these patients, reaction products were localized to the lamina lucida of the basement membrane zone, the same location as those immunoreactants bound in vivo in tissue from patients with bullous pemphigoid [3,4], herpes gestationis [5], and some patients with dermatitis herpetiformis [6]. As details were available on only 1 of these patients [2], we sought to determine the ultrastructural localization of in vivo-bound immunoreactants in a larger group of patients with cicatricial pemphigoid. In addition, we examined the sera of these patients for the presence of circulating autoantibodies and complement-fixing factors and have also, in 2 patients, examined the location of in vivo-bound immunoreactants by direct immunofluorescence following mechanical suction blister induction.

MATERIALS AND METHODS

Patients

Ten patients (6 male, 4 female), ranging in ages from 36–84 years (mean, 60 years), met the clinical criteria for cicatricial pemphigoid and were included in this study. The diagnosis of cicatricial pemphigoid was made in those patients whose ocular findings included symblepharons and conjunctival blisters, edema, and erythema. Oral involvement was characterized by the presence of blisters, erosions, or ulcerations within the oral cavity; marked gingivitis was also present in about half of our patients. Skin involvement was characterized by the development of tense vesicles or bullae. Eight of 10 of the patients had positive immunofluorescence findings (linear deposition of immunoglobulins and/or C3 along the basement membrane zone) in association with lesional involvement of oral and/or ocular mucosa. Two patients were included who had negative direct immunofluorescence findings but who had evidence of active blister formation or symblepharons within ocular tissue as well as recurrent oral lesions.

Clinical findings are summarized in Table I. In all patients having skin involvement, lesions were far less frequently seen on skin than on mucosal surfaces. Two patients had laryngeal involvement with resultant scar formation as visualized by direct laryngoscopy; associated symptoms included intermittent aphonia and dyspnea. Esophageal disease was also present in 2 patients, in 1 patient manifested by stricture formation and in the other, by upper gastrointestinal hemorrhage.

Tissue Specimens Examined

Three-millimeter punch biopsies were obtained from clinically uninvolved buccal mucosa of 9 of our subjects. Punch biopsies were also obtained from skin of 9 patients; 7 specimens were taken from normal skin (as these patients had no active skin lesions) while in 2 patients, biopsies were obtained from perilesional normal-appearing skin. In 2 patients (1 of whom had ocular involvement), specimens of conjunctiva were kindly provided by Drs. R. Nussenblatt and A. Palestine (National Eye Institute). Half of each specimen was directly embedded in O.C.T. compound (Lab-Tek Products, Naperville, Illinois) and snap-frozen; these specimens were subsequently utilized for direct immunofluorescence studies. The remaining halves of the tissues were placed in 10% glycerin (in phosphate-buffered saline PBS, pH 7.4) solution, refrigerated at 4°C for 2 h, subsequently placed in O.C.T. embedding compound, and then slowly frozen. Specimens prepared in the latter manner were then utilized for immunoperoxidase staining and subsequently processed for immunoelectron microscopic examination.

Serum

Serum specimens were obtained from 8 patients; these were stored at −70°C until used for indirect immunofluorescence and complement fixation studies.

Immunofluorescence Studies

Direct immunofluorescence: Six micrometer-thick cryostat sections of unfixed tissues were utilized for direct immunofluorescence testing using fluoresceinated polyclonal antibodies (Cappel Laboratories, West Chester, Pennsylvania) directed against human IgG (1:80 dilution), IgA (1:40), IgM (1:20), IgE (1:20), C3 (1:40), and fibrin (1:40). All such dilutions were prepared in PBS (pH 7.4).

Indirect immunofluorescence: Indirect immunofluorescence testing was performed on sera from 8 patients with cicatricial pemphigoid utilizing both normal human skin and normal oral mucosa as substrates. Six micrometer-thick cryostat sections were prepared using these two substrates, and undiluted and serially diluted sera (1:2–1:16) were applied to the tissue sections. Normal human sera were used at identical dilutions as controls. Following a 30-min incubation at room temperature in a moisturized chamber, the slides were washed 3 times in PBS (pH 7.4), and then incubated with fluoresceinated polyclonal antibodies to human IgG or IgA (1:40 dilution). The slides were then washed in PBS 3 times, mounted with a 50% glycerin (in PBS) solution and examined by fluorescence microscopy using epi-illumination.

Complement fixation: Sera from 8 patients with cicatricial pemphigoid were examined for the presence of a complement-fixing factor

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Abbreviations:
PBS: phosphate-buffered saline

TABLE I. *Cicatricial pemphigoid: patient summary*

Patient	Age	Sex	Duration (yr)	Distribution of involvement			
				Oral	Ocular	Cutaneous	Other
1	57	M	18	+	−	−	Larynx, esophagus
2	65	M	3	+	+	Arms, legs (rare)	Larynx
3	68	M	4	+	−	Hands, scalp	−
4	76	M	15	+	+	Upper back, face	−
5	56	F	14	+	+	Perineal (rare)	−
6	50	F	2½	+	−	−	−
7	49	F	1	+	−	Widespread	−
8	36	M	7	−	+	Ankles, waist (rare)	−
9	64	M	2	+	+	Perianal	Esophagus (stricture)
10	84	F	1	+	−	Umbilical (rare)	−

TABLE II. *Cicatricial pemphigoid: direct immunofluorescence*

Patient	Buccal mucosa						Skin[a]					
	Ig				C3	Fibrin	Ig				C3	Fibrin
	G	A	M	E			G	A	M	E		
1[b]	+	−	−	−	+	−	−	−	−	−	−	−
2	+	−	−	−	+	−	+	−	−	−	−	−
3	+	−	−	−	+	−	+	−	−	−	+	−
4							+	+	−	−	+	−
5	−	−	−	−	−	−	−	−	−	−	−	−
6	+	+	−	−	−	−	−	−	−	−	−	−
7	−	−	−	−	+	−						
8[b]	+	+	+		+	+	−	+	−	−	+	−
9	−	−	−	−	−	−	−	−	−	−	−	−
10	−	+	−	−	−	−	−	−	−	−	−	−

[a] Perilesional skin, patients #3 and #4; clinically normal skin, all others.
[b] Direct immunofluorescence performed on conjunctiva:
Patient #1 positive for IgG, C3, fibrin;
Patient #8 positive for IgA, C3, fibrin.

using a modification of the previously described procedure for indirect immunofluorescence [7].

Immunoperoxidase Staining

Tissue specimens from 6 patients having positive direct immunofluorescence were stained using an immunoperoxidase technique and processed for subsequent electron microscopic evaluation. In 5 patients, clinically uninvolved buccal mucosa was utilized. In 1 patient, perilesional skin was used and in 1 of the patients having detectable immunoreactants in both buccal mucosa and conjunctiva, conjunctiva was also stained. Because of the varied composition of immunoreactants in the tissues of these patients and also their relative amounts as determined by direct immunofluorescence, several different protocols were used. Dilutions of all immunoreagents were made in 1% bovine serum albumin in PBS (pH 7.4). In some patients having relatively large amounts of in vivo-bound IgG and/or C3, peoxidase staining was performed by applying horseradish peroxidase-conjugated staphylococcal protein A (1:5–1:10 dilution; Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Maryland) or goat antihuman C3 (1:5–1:10) followed by horseradish peroxidase-conjugated staphylococcal protein A (1:5–1:10). Other tissue specimens were incubated sequentially with normal rabbit serum (1:10) (to decrease background), goat antihuman IgA, IgG, or C3 (1:20–1:40), rabbit antigoat IgG (1:5), and goat peroxidase-antiperoxidase (1:5–1:100, Cappel Laboratories). Following the final incubation with either horseradish peroxidase-conjugated staphylococcal protein A or goat peroxidase-antiperoxidase, the slides were washed in 0.2 M Tris HCl buffer (pH 7.6) and then incubated for 20 min in a solution containing diaminobenzidine (50 mg, Sigma Chemical Co., St. Louis, Missouri) and 30% hydrogen peroxide (100 μl) in 10 cc of 0.2 M Tris HCl buffer (pH 7.6). Following incubation, the slides were rinsed in PBS (pH 7.4) and then processed for electron microscopy. As controls, normal human buccal mucosa was reacted with the same reagents in an identical manner.

Electron Microscopy

The tissue sections were prepared as previously described [5] and subsequently viewed with a 400 T Philips electron microscope.

Mechanical Suction Blister Induction

Mechanical suction blisters were induced on the upper arms of 2 of the 4 cicatricial pemphigoid patients known to have in vivo-bound immunoreactants in clinically uninvolved skin. The methodology for production of such blisters has been described previously [8]. Following induction of a small blister (1–2 mm), a 4-mm punch biopsy was performed in a manner identical to that described previously for direct immunofluorescence. Six micrometer-thick cryostat sections were obtained and tissues were first incubated with bullous pemphigoid serum (1:10–1:20 dilution) or normal human serum (1:10–1:20). Following a 30-min incubation and subsequent washes in PBS, the slides were then incubated with fluoresceinated goat antihuman IgG (1:40). Following incubation and final washings, the slides were mounted as previously described and examined by fluorescence microscopy.

RESULTS

Immunofluorescence Studies

Direct immunofluorescence of clinically normal-appearing buccal mucosa revealed the presence of immunoreactants along the basement membrane zone in specimens from 8 of 10 patients with cicatricial pemphigoid (Table II). IgG and C3 were the most commonly bound immunoreactants, each being present in mucosa of 5 of the 10 patients studied. Four of these 5 patients had both IgG and C3 present. Skin was also examined by direct immunofluorescence in 9 of these 10 patients; in 2, biopsies were obtained from perilesional sites. Only 4 of 9 had

evidence of in vivo-bound immunoreactants within skin; 2 of these 4 positive biopsies were from perilesional skin. IgG and C3 were most commonly seen, each being present in 3 of 9 patients. In 3 patients, immunoreactants were present in buccal mucosa but not in skin. In those patients having immunoreactants in both buccal mucosa and skin, the reaction products appeared to be present in both greater amounts and variety in buccal mucosa than in corresponding skin. In 1 of the 2 patients (# 1) in whom conjunctiva, skin, and buccal mucosa were biopsied, the conjunctiva had intense staining for fibrin as well as weaker staining for IgG and C3; fibrin was absent in his buccal mucosa and skin. Interestingly, this patient lacks clinical evidence or history of eye involvement by this disease. In the other patient (# 8) in whom buccal mucosa, skin, and conjunctiva were all examined by direct immunofluorescence, the intensity and variety of the in vivo-bound immunoreactants found in conjunctiva (IgA, C3, fibrin) appeared intermediate as compared to those in buccal mucosa and skin. In 2 patients multiple biopsies from buccal mucosa and skin were negative by direct immunofluorescence; this despite the fact that in both of these patients, conjunctiva and oral mucosa were clinically involved with recurrent blister formation and erosions. In 1 of these 2 patients, symblepharons were also present. Therefore both of these patients were felt to meet clinical criteria for the diagnosis of cicatricial pemphigoid despite negative immunofluorescence findings.

Results of indirect immunofluorescence demonstrate that in only 1 patient (of the 8 tested) was a low titer (1:10) of a circulating anti-basement membrane zone antibody (of the IgA class) detectable using normal human skin as substrate. Tissues from this patient showed a very bright fluorescence of buccal mucosa with antihuman IgA. In the sera of all patients examined, circulating upper epidermal cytoplasmic antibodies were detectable in low titers. When normal human buccal mucosa from 3 different volunteers was used as the substrate for indirect immunofluorescence, no specific binding along the basement membrane zone was seen.

Sera from 8 patients with cicatricial pemphigoid were examined by indirect immunofluorescence for the presence of a complement-fixing factor similar to that detectable in some patients with herpes gestationis. Normal human skin was used as the tissue substrate. No such factor was detectable using any of these sera.

Immunoelectron Microscopy

Specimens from 6 patients with cicatricial pemphigoid having positive direct immunofluorescence of buccal mucosa and/or skin were stained by the immunoperoxidase technique and subsequently processed for immunoelectron microscopy. Buccal mucosa was tested in 5 of these patients; in an additional patient, perilesional skin was used. Immunoperoxidase staining was also performed on conjunctiva obtained from a patient who had in vivo-bound immunoreactants detectable in conjunctiva, buccal mucosa, and clinically uninvolved skin. In all patients and tissues examined, In vivo-bound immunoreactants (IgG, IgA, C3) were localized within the lamina lucida of the basement membrane zone (Figs 1–3). In no tissue was any reaction product detectable within either the lamina densa or the sublamina densa region.

Mechanical Suction Blisters

Mechanical suction blisters were induced in 2 patients known to have in vivo-bound IgG within the basement zone of clinically normal skin. These blisters were biopsied and sections subsequently incubated with normal human serum or bullous pemphigoid antibodies directed against an antigen within the lamina lucida of the basement membrane. When normal human serum was incubated with this tissue and then a fluoresceinated antihuman IgG subsequently used as the second step, no stain-

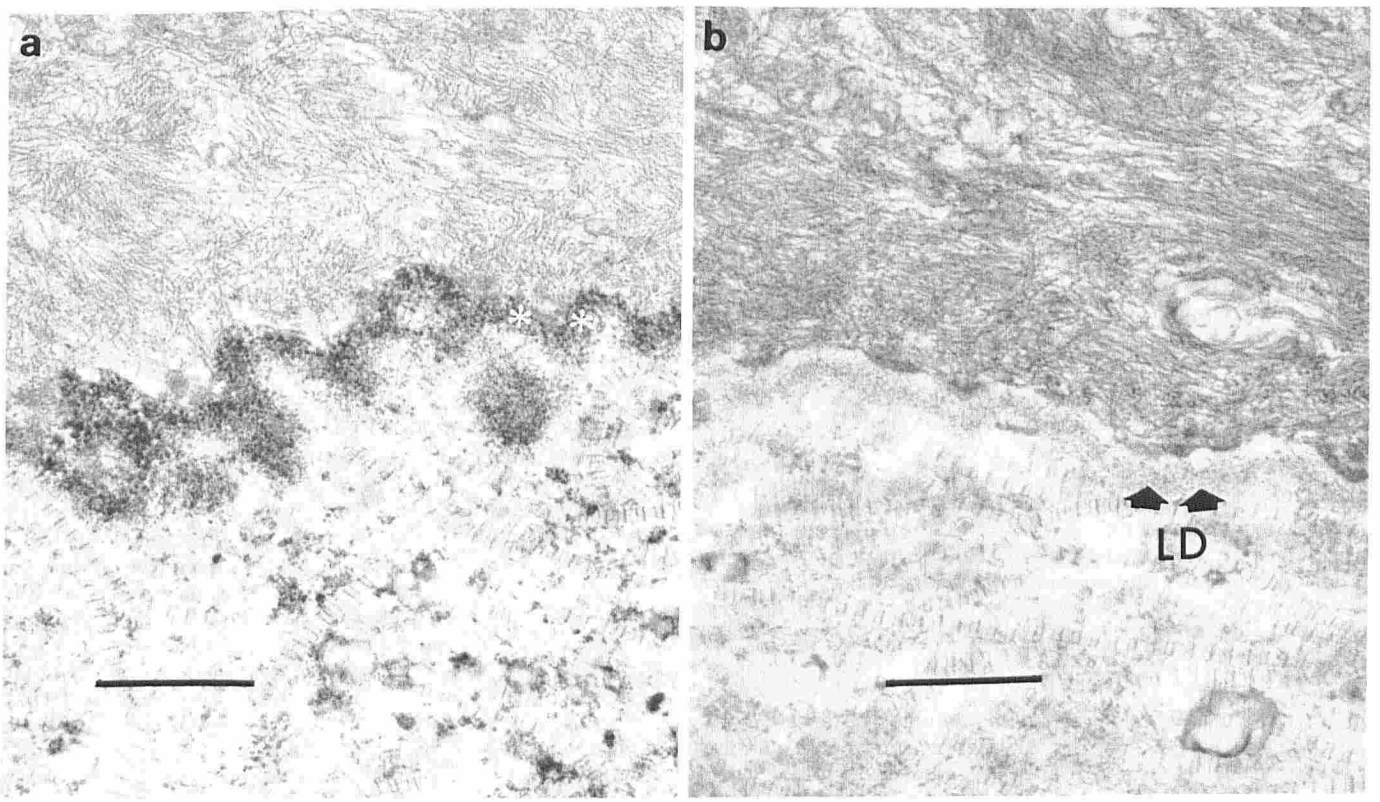

FIG 1. *a*, Immunoelectron micrograph of buccal mucosa of patient #8 showing in vivo-bound IgA within the lamina lucida (asterisks). *b*, Negative control of same tissue using normal goat serum instead of the goat antihuman IgA reagent. *LD* = lamina densa. *Bars* = 0.5 μm.

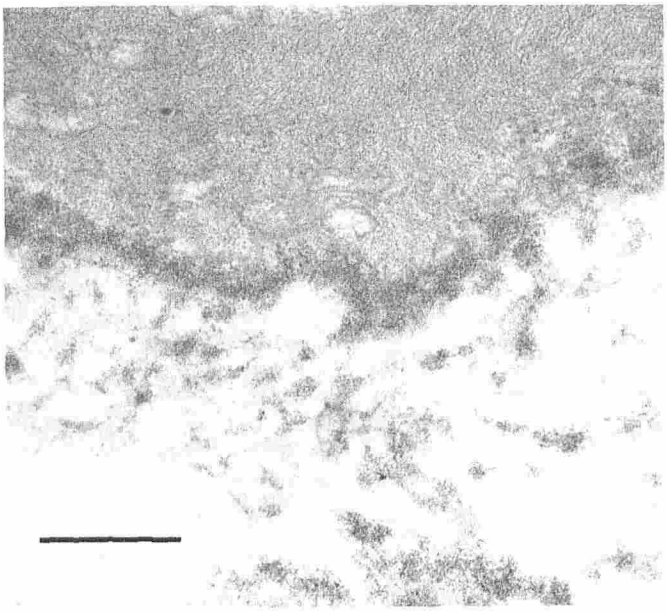

FIG. 2. Immunoelectron micrograph of buccal mucosa of patient #2 showing in vivo-bound IgG within the lamina lucida. *Bar* = 0.5 μm.

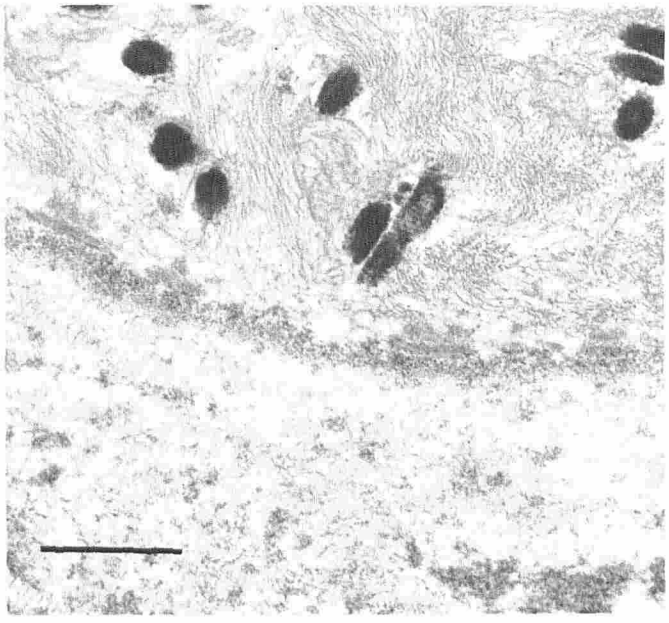

FIG. 3. Immunoelectron micrograph of perilesional skin of patient #4 showing in vivo-bound IgA within the lamina lucida. *Bar* = 0.5 μm.

ing was detectable along the roof of the blister although staining was visualized along the base, due to the staining of the in vivo-bound IgG in the tissue. Using serum from a patient with bullous pemphigoid, there was clear staining of the roof of the blister, suggesting that the bullous pemphigoid antigen and the cicatricial pemphigoid antigen are distinct.

DISCUSSION

Cicatricial pemphigoid is an uncommon bullous disease characterized by the development of blisters and scar formation within mucous membranes, particularly eye and oral cavity. Less frequently, patients may develop subepidermal blisters in skin. As opposed to bullous pemphigoid, patients with cicatricial pemphigoid infrequently have detectable circulating anti-basement membrane zone antibodies within their sera.

In the literature, approximately 80–97% of patients meeting clinical criteria for cicatricial pemphigoid are reported to have positive direct immunofluorescence of oral mucosa [9–12]. This is in agreement with our findings of positive direct immunofluorescence in 8 of 10 patients. Circulating anti-basement membrane zone antibodies have been seen in approximately 26–36% of patients with this disease [10,11]; if present, they are usually detectable in only low titers. In our series, only 1 of 8 patients tested had detectable circulating antibodies. This is in contrast to bullous pemphigoid in which the sera of approximately 80% of patients examined by indirect immunofluorescence are found to have circulating anti-basement membrane zone antibodies [10]. One possible reason why circulating anti-basement membrane zone antibodies have not been detected more frequently in patients with cicatricial pemphigoid is that a potentially less favorable tissue substrate (i.e., skin) is frequently used in these studies [11]. Considering the predilection for mucosal involvement in this disease, it has been postulated that circulating anti-basement membrane antibodies in these patients may have more specificity in binding normal oral mucosa than normal human skin. In the present study, we were unable to detect specific basement membrane zone binding to normal oral mucosa by sera from 8 of our patients. We have additionally examined sera of our patients for the possible presence of a circulating anti-basement membrane zone complement-fixing factor. As opposed to herpes gestationis where such a serum factor may be often detected [7], we have been unable to demonstrate a complement-fixing factor within any cicatricial pemphigoid sera that we have yet tested.

Prior to this study there have been only 2 patients with cicatricial pemphigoid in whom immunoelectron microscopy has been reported [1,2]. In both of those patients, lamina lucida deposition was noted. This is in agreement with our experience with 6 patients meeting clinical criteria for this disease. In buccal mucosa of 5 patients, in vivo deposition of immunoglobulins and/or complement was detected exclusively within the lamina lucida of the basement membrane zone. In addition, we have found the same ultrastructural localization of immunoreactants in perilesional skin and in conjunctiva of such patients. This then confirms that cicatricial pemphigoid should be classified with bullous pemphigoid, herpes gestationis, and some forms of dermatitis herpetiformis as a bullous disease characterized by the presence of *in vivo*-bound immunoglobulins and/or complement within the lamina lucida. It is of interest that despite the ultrastructural similarities in these diseases, scarring is seen only in patients with cicatricial pemphigoid. It is unknown why this phenomenon is selective for this disease.

By induction of mechanical suction blisters in 2 cicatricial pemphigoid patients, we have been able to evaluate the localization of their in vivo-bound IgG in a slightly different manner. It has been well demonstrated previously that mechanical suction blisters reliably produce a cleavage plane within the lamina lucida [13] and that the bullous pemphigoid antigen is found primarily along the roof of the blister [14]. When blisters are induced in patients with bullous pemphigoid, the in vivo-bound immunoreactants are ordinarily on the roof of the blister [15]. When we incubated these two tissues from cicatricial pemphigoid patients with bullous pemphigoid serum, we found very strong staining primarily along the roof as well as staining along the blister base; the latter staining was due presumably to the in vivo-bound antibody. In contrast, when we used normal serum as the first step in place of pemphigoid serum, we observed fluorescent staining only along the blister base, suggesting that the antigen to which these antibodies are bound in cicatricial pemphigoid resides within a lower portion of the lamina lucida than that containing bullous pemphigoid antigen. These findings suggest that different structural antigens located within the same ultrastructural region of the basement membrane zone, the lamina lucida, may be involved in the pathogenesis of cicatricial and bullous pemphigoid.

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